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APPLICATION NOTE

Combining protein extraction using the Omni Bead Ruptor Elite bead mill homogenizer with detection of phosphorylation events in mouse kidney samples using AlphaLISA immunoassay.

Summary

The Omni Bead Ruptor Elite™ bead mill homogenizer enables rapid and efficient tissue homogenization for various sample types. It can homogenize samples using optimized bead matrices to facilitate sample disruption. Bead milling can replace traditional disruption methods, including incubation in the presence of harsh chemicals or pulverization under cryogenic conditions [1].

5-Fluorouracil (5-FU) is an antimetabolic agent and influences the synthesis of DNA and RNA in normal and tumor cells. 5-FU is widely used in chemotherapy for various colon, breast, gastrointestinal, head, neck, and pancreatic cancers [2]. Considered to be a nephrotoxic compound, toxicity and unwanted side effects occur following its use.

In this application note, female nude mice were treated with 5-FU to generate a kidney toxicity model and study the cellular effects of 5-FU treatment. Mouse tissue samples were processed for total protein lysates using the Omni Bead Ruptor Elite bead mill homogenizer and tested for biomarkers using AlphaLISA® *SureFire® Ultra* technology, investigating the expression level of both phosphorylated and total targets. One of the targets investigated was extracellular signal-related kinase (ERK), which is found to be involved in acute and chronic kidney disease [3].

For research use only. Not for use in diagnostic procedures.

Omni Bead Ruptor Elite bead mill homogenizer

Materials and methods

Equipment

- Omni Bead Ruptor Elite bead mill homogenizer (Cat # 19-042E)
- Eppendorf Refrigerated Centrifuge 5418R (VWR, Cat # 97058-928)
- EnVision 2105 Multimode Plate Reader (Cat # 2105-0010)

Materials

- 2 mL Hard Tissue Homogenizing Mix 2.8 mm Ceramic Beads (Cat # 19-628)
- AlphaLISA *SureFire Ultra* kits – Phosphorylated ERK (Thr202/Tyr204) (Cat # ALSU-PERK-A), and Total ERK (Cat # ALSU-TERK-A)
- AlphaLISA *SureFire Ultra* Lysis Buffer (Cat # ALSU-LB-100ML)
- Roche Complete Protease Inhibitor mini-tablet, EDTA free (Millipore Sigma, Cat # 5892791001) and Roche PhosSTOP Phosphatase Inhibitor (Millipore Sigma, Cat # 4906837001)
- Eppendorf Tubes – LoBind Protein, 2 mL size (VWR, Cat # 80077-226)
- Pierce Rapid Gold BCA Protein Assay Kit (Thermo Fisher, Cat # A53226)
- Assay Plates – BCA Protein assay: Costar 3610 White 96-well clear bottom assay plate (VWR, Cat # 29444-010), and AlphaLISA *SureFire Ultra* assay plate: ProxiPlate-384 Plus, White 384-shallow well Microplate (Cat # 6008280)

Procedure

Female nude mice were obtained from Charles River Laboratory and separated into two groups, untreated and drug-treated. The drug-treated group was subjected to an intermittent dose of 5-fluorouracil (5-FU) treatment at 100 mg/kg three times per week for 2 weeks. Mouse tissues were harvested 6 hours after the final drug dose in week two. Tissues were flash-frozen and stored in the LN_2 repository until processing. On the day of protein processing, tissues were pulled from LN_2 storage and placed on ice.

Kidney tissues were weighed and rinsed twice in ice-cold PBS to remove residual blood from the dissection process. Whole kidneys weighing 150-200 mg were transferred to 2 mL reinforced tubes pre-filled with 2.8 mm ceramic beads (Cat # 19-628). Before homogenization, 500 µL of ice-cold 1X AlphaLISA *SureFire Ultra* Lysis Buffer supplemented with protease and phosphatase inhibitors was added per 100 mg of tissue. The Omni Bead Ruptor Elite bead mill homogenizer (Figure 1) was set to 4.5 m/sec for 30 seconds and run for 1 cycle. Immediately following homogenization, samples were transferred to pre-chilled Eppendorf tubes and centrifuged at 14,000 RPM for 10 min at 4 °C to separate soluble protein from tissue debris. After centrifugation of the samples, the soluble protein supernatants were transferred to new chilled Eppendorf tubes to determine the total protein concentration.

The Pierce Rapid Gold BCA Protein Assay was performed following the manufacturer's protocol alongside the BSA standard curve. After the total protein concentration was interpolated from the standard curve, each sample was diluted to 5 mg/mL in a new Eppendorf tube and frozen at -80 °C until they were processed by AlphaLISA immunoassay. The total protein concentration from each sample is shown in Table 1.

AlphaLISA *SureFire Ultra* Assays were performed for phosphorylated ERK (Thr202/Tyr204) and total ERK signal for normalization following the standard protocol. 10 µL of each sample was tested in duplicate in the assay at three concentrations of total protein to ensure the AlphaLISA signal would be in the linear range of the assay – 1 mg/mL, 0.5 mg/mL, and 0.25 mg/mL. Results are shown for the middle concentration of total protein, 0.5 mg/mL. AlphaLISA technology is a fast, highly sensitive, homogeneous, no-wash assay platform that can be performed in a microplate format. AlphaLISA assays require two bead types: Donor beads and Acceptor beads. In the AlphaLISA SureFire Ultra assay, Donor beads are coated with streptavidin to capture one of the detection antibodies, which is biotinylated. Acceptor beads are coated with a proprietary CaptSure™ agent that immobilizes the other antibody, labeled with a CaptSure tag. In the presence of target protein, the two antibodies bring the Donor and Acceptor beads close together. Upon excitation at 680 nm, a photosensitizer inside the Donor bead converts ambient oxygen to an excited singlet state. Singlet oxygen diffuses up to 200 nm

to produce a chemiluminescent reaction in the Acceptor bead, leading to light emission at 615 nm. If an Acceptor bead is not in proximity to a Donor bead, little to no signal is produced over background. AlphaLISA signal was measured on the EnVision 2105 Multimode Plate Reader (Figure 3) using pre-programmed settings for Alpha with excitation at 680 nm and emission at 615 nm. A schematic of the AlphaLISA *SureFire Ultra* assay for a phosphorylated target is shown in Figure 2.

Figure 1: Omni Bead Ruptor Elite bead mill homogenizer

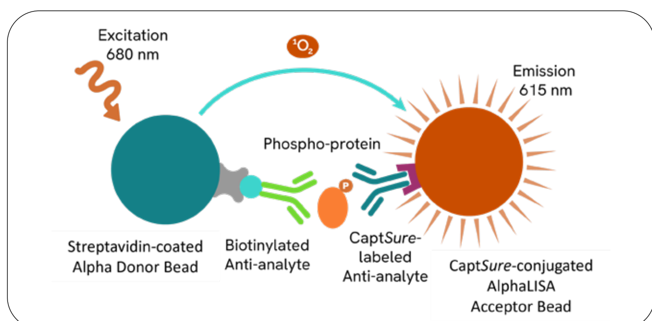

Figure 2: AlphaLISA *SureFire Ultra* assay schematic

Figure 3: EnVision 2105 multimode plate reader

Results

Mouse kidney tissue samples were processed as described above, and total protein concentration was normalized before performing the phosphorylated ERK and total ERK AlphaLISA *SureFire Ultra* assays. Total protein amounts from each mouse sample are shown in Table 1 as determined by the BCA Protein Assay.

Table 1: Protein harvest results from each mouse.

Mouse	Treatment	Concentration (mg/mL)
1	Control	18.94
2	Control	20.78
3	Control	23.61
4	100 mg/kg 5-FU	21.09
5	100 mg/kg 5-FU	21.58
6	100 mg/kg 5-FU	25.57

Figure 4 shows the normalized AlphaLISA signal for the ERK assays. The signal from the phosphorylated ERK assay was divided by the total ERK result to normalize each sample. The treatment course of 5-FU drug led to a slight decrease in ERK phosphorylation on markers Thr202 and Tyr204. There was an approximate 20 % decrease relative to control-treated mice when comparing the mean normalized signal of each treatment group. This change was small but significant (p-value of 0.0059) and reproducible, as Figure 4 represents two experiments with the same intermittent dosing regimen. The decrease was not due to a change in total ERK signaling as the average total ERK AlphaLISA signal was 1628.3 for Untreated and 1626.8 with 5-FU treatment.

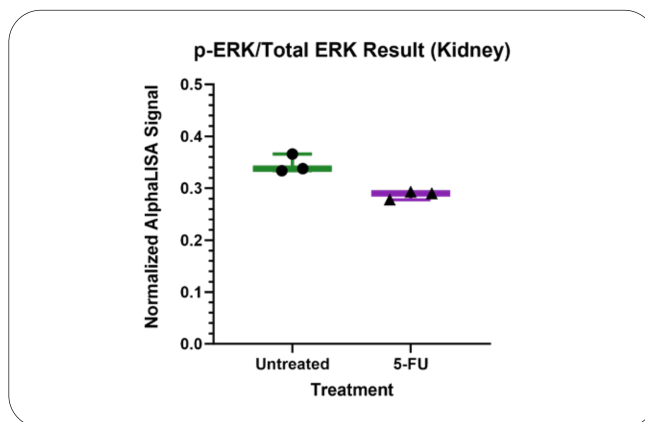

Figure 4: Normalized phosphorylated ERK AlphaLISA result. 3 mice per treatment condition. There was a slight decrease in phosphorylated ERK signaling in 5-FU treated mice relative to untreated control mice.

Conclusions

The Omni Bead Ruptor Elite bead mill homogenizer is capable of homogenizing soft tissue samples, such as mouse kidneys, in 30 seconds to obtain adequate protein yields for the AlphaLISA Immunoassay, which requires only 10 µL of sample at or below 1 mg/mL concentration of total protein. In our experiments, we saw a slight change in phosphorylation status on ERK at Thr202 and Tyr204 markers in mouse kidneys but no change to the total ERK signal after intermittent dosing of mice with 5-FU drug for two weeks. Furthermore, histological staining on the kidney tissues showed no change, suggesting the 5-FU dosing regimen was insufficient to cause acute kidney damage in the mice after two weeks of treatment. This result demonstrates that the Omni homogenization system is well suited for downstream protein applications and can be paired with AlphaLISA technology to track biomarker expression in drug-treated mice.

References

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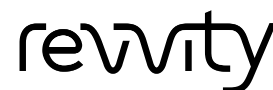

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